FREQUENCY-DEPENDENT SELECTION FOR PLASMID-CONTAINING CELLS OF *ESCHERICHIA COLI*[1]

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ABSTRACT

Colicin-producing plasmid-containing cells of *E. coli* exhibit frequency-dependent selection when grown in glucose-limited continuous culture with the corresponding plasmid-free strain. The bases of this frequency-dependent effect are shown to be (1) the lower growth rate of the plasmid-containing strain under these conditions, and (2) the production of colicin, which attenuates the growth rate of the plasmid-free strain. These results are discussed in relationship to the maintenance of genetic variation in prokaryotes.

THERE is now abundant evidence that natural populations of bacteria, in common with eukaryotes, are highly variable genetically. One of the most striking manifestations of this variability is seen in the variety and frequency of occurrence of plasmids in nature. NOVICK (1974) in a review article documented the existence of 269 different naturally occurring bacterial plasmids in gram-negative bacteria. Undoubtedly this figure represents an extremely small proportion of all plasmids, since there have been few systematic studies designed to detect and isolate bacterial plasmids. Until recently, plasmids have been detected largely by chance, generally because many confer transmissible resistance to one or more antibiotics or other inhibitory agents. There are now convenient methods for detecting these R factors or other small circular DNA molecules that allow the detection of plasmids having no obvious effect on cell phenotype (RADLOFF, BAUER and VINOGRAD 1967; COZZARELLI, KELLY and KORNBERG 1968; GUERRY, LEBLANC and FALKOW 1973).

Not only is the diversity of plasmids high, but their distribution in populations is widespread. FREDERICQ (1963) has estimated that 50% of all strains of Enterobacteriaceae contain a bacterial plasmid, of which half are of the bacteriocin-producing variety. In one of the few systematic studies undertaken, FALKOW and SKERMAN (see FALKOW 1975) found that 95 out of 252 naturally occurring strains of *Escherichia coli* isolated from an unacculturated population in Northern Queensland, Australia, contained a conjugative plasmid. Nor is this genetic variability in bacterial populations confined to the level of plasmids. MILKMAN (1973) has detected a large number of isozyme variants for five loci in naturally occurring strains of *E. coli* independently isolated from diverse sources.

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The prevalence of R-type plasmids can probably be explained to a large extent by the widespread use of antibiotics during the past several decades (FALKOW 1975). However, the use of antibiotics does not explain all the genetic variability for plasmids. Both the frequency with which bacterial plasmids are found and their diversity suggest that they confer other selective advantages on their bacterial hosts. Our results suggest that cells containing bacteriocinogenic plasmids may be selected for in certain environments where the concentration of bacteriocin is above a critical level.

We have undertaken a systematic comparison of plasmid-free and plasmid-containing cells of *E. coli* in an attempt to understand the roles of the plasmid in determining both the growth dynamics and the evolution of the bacterium. In this paper, we report on the selective characteristics of the bacteriocin (colicin) ColE1-producing plasmid RSF2124 (So, GILL and FALKOW 1975) with a view to explaining the occurrence of such plasmids in nature. This plasmid was chosen because it is a hybrid plasmid containing the DNA of ColE1 plus the transposon TnA, which, by virtue of the inverted repeat consequences found at its termini, promotes recombination. The TnA sequence carries a gene for β-lactamase, which confers resistance to ampicillin. This plasmid can be considered to possess properties of both colicin-producing plasmids and R-type plasmids; thus, it is particularly suitable for any study aimed at explaining the maintenance of plasmids in bacterial populations. The small size (11.3 kilobase pairs), favorable copy number (10 to 30 copies per cell) and its drug-resistance gene make this plasmid amenable for laboratory manipulation. Furthermore, the frequent use of RSF2124 as a cloning vehicle for foreign DNA segments (*e.g.*, LOMAX *et al.*, 1977) lends added significance to these studies.

MATERIALS AND METHODS

Strains: All bacteria used were derivatives of *E. coli* K12 strain RH202 (Table 1) constructed by transduction with bacteriophage P1bt (LAKSHMI and HELLING 1976) or by transformation (LEDERBERG and COHEN 1974). Plasmids pML17, pML27, and pML31 are derivatives of RSF2124 (So, GILL and FALKOW 1974) that contain *Euglena gracilis* chloroplast DNA segments

TABLE 1

Bacterial strains

Strain	Genotype	Origin
RH201	F- *thr1 leu5 thi1 lacY1 tonA21 supE44 hss1* or (*hsm1* + *hsr1*)	Genetic stock center CGSC 5346; M94 of MESELSON and YUAN (1968)
RH202	as RH201 but *thr*+ *leu*+	Transduction
RH204	as RH202 but *araAh9*	Transduction of RH201, selection for *thr*+ and *leu*+, co-transfer of *araAh9*
RH250	as RH202 plus plasmid RSF2124	Transformation of RH202
RH251	as RH202 plus plasmid pML17	Transformation of RH202
RH256	as RH202 plus plasmid pML27	Transformation of RH202
RH268	as RH202 plus plasmid pML31	Transformation of RH202

generated by endonuclease R-EcoRI (segments P, R, and W in pML17, H in pML27, and K in pML31; LOMAX *et al.* 1977; HELLING and LOMAX, unpublished). The sizes of these plasmids in kilobase pairs are: RSF2124, 11.3; pML17, 16.2; pML27, 16.0; and pML31, 14.5.

Media: The minimal medium was that of DAVIS and MINGIOLI (1950), but lacking citrate. Thiamine·HCl was added to 1 μg/ml. Minor salts were added to the medium for some of the chemostat experiments as previously described (ADAMS and HANSCHE 1974). Glucose was added as a carbon source at a concentration of 0.2% (w/v) for the unlimited growth experiments, and 0.025% (w/v) for the chemostat experiments. This latter glucose concentration gave a population density of about 1.0×10^8 cells per ml at our dilution rates. Glucose and thiamine·HCl were sterilized by filtration through nitrocellulose membranes with 0.22 μm pore size prior to use in chemostat medium. After autoclaving the medium, filter-sterilized ampicillin was added when necessary to a concentration of 40 μg per ml for the solid medium and 20 μg per ml for the chemostat medium. Viable counts were determined on tryptone agar (TA).

Growth and sampling: Unlimited growth cultures on minimal medium were grown at 37° in a gyratory shaker at 150 to 200 gyrations per minute. Chemostat experiments were maintained at 37° in chemostats of various sizes from 150 to 180 ml. Dilution rates were between 0.35 hr^{-1} and 0.27 hr^{-1}, which corresponded to cell generation times of between two and 2.5 hr. Population density was monitored by the optical density of the culture at 550 nm. Two different techniques were used for initiating the competition experiments: (1) the two strains were grown to numerical equilibrium in separate chemostats at the same dilution rates, and then one chemostat was inoculated with cells from the second. Wash out was briefly interrupted in order to allow the cells to mix. This procedure was found necessary for most competition experiments in order to control precisely the initial frequencies of the competing strains. (2) One strain was grown to numerical equilibrium in a chemostat, and at the start of the experiment this chemostat was inoculated with a few mls of an exponentially growing or early stationary-phase batch-culture of the second strain. This procedure was found to be satisfactory when the initial frequency of the second strain was required to be low (< 0.02). Generally, the cells containing a plasmid were grown in the presence of ampicillin (20 μg per ml) until medium flow was started in order to ensure that all cells contained the plasmid at the beginning of the experiment. This was especially important for certain derived plasmids, such as pML27, that segregate plasmid-free cells at a significant rate. It was not necessary to remove the ampicillin from the medium before the addition of the plasmid-free cells, as most of it was degraded by the β-lactamase of the plasmid-containing cells. The remainder was rapidly diluted out when medium flow was initiated. Changes in the frequency of plasmid-containing cells were monitored over time by plating samples of cells onto TA plates and replicating these onto TA plus ampicillin plates. All time was measured in cell generations (ln 2/dilution rate, KUBITSCHEK 1970). Colicin sensitivity was determined by stabbing RH250 into TA plates, allowing growth overnight, killing the surface cells by exposure to chloroform vapor for five minutes, and overlaying with 3 ml of soft agar containing approximately 5×10^7 cells of a test strain. Sensitive strains could be identified after six to eight hrs of incubation at 37° by a clear spot of no growth around the stab. Resistant strains showed no such growth inhibition.

Biohazard consideration: All experiments involving recombinant DNA molecules were carried out under P2-level conditions as specified in "*Guidelines for research involving recombinant DNA molecules*" [Fed. Register **41**; (131), 27913, 1976], and as approved by the local biohazard committee.

RESULTS

No difference in unlimited growth rates of plasmid-free and plasmid-containing strains: As an initial test for the selective effect of a bacterial plasmid, growth rates of plasmid-free cells (RH202) and cells containing the plasmid RSF2124 (RH250) were measured in a minimal salts + glucose medium, in batch culture. Under these conditions all components of the medium are in excess (unlimited

culture). The results shown in Table 2 indicate that the growth rates of the two strains are almost identical and there is no detectable effect of the plasmid RSF2124 on growth rate. The growth rates of strains HR256 and RH268 containing derived plasmids pML27 and pML31, respectively, were slightly lower, although these differencies were not significant. Thus, replication of extra DNA in the plasmid-containing cells has no obvious detrimental effect on growth rate. Under nonlimiting growth conditions, all essential components of the medium are in excess, and the lack of a growth rate difference is perhaps not surprising. The effect of the plasmid RSF2124 on the fitness of the bacterial host cell was therefore investigated under conditions of carbon limitation, where the effect of the increased amount of DNA to be replicated may be manifested in a reduced fitness.

Frequency-dependent selection under conditions of carbon limitation: Cells were grown in chemostats with glucose as the growth-limiting factor. Growth of the two strains (RH202 and RH250) together in a single chemostat allows a precise determination of relative fitness. In this environment, the relative fitness of the plasmid-containing strain was dependent on the initial frequency with which it was introduced into the chemostat (Figure 1). Above an initial frequency of 0.5, the plasmid-containing strain (RH250) was selected for and replaced the plasmid-free strain (RH202) in the chemostat, whereas below this frequency the reverse happened. This result was unexpected and could be due to differences in growth rate between the two strains in mixed culture, not observed when cultured singly.

Mechanism of frequency dependency: We must postulate the action of two different factors to produce such a frequency dependency: (1) there must be a growth-rate difference between the two strains and (2) under certain conditions this difference must be reversed by the action of a second factor. The simplest explanation of the frequency-dependent effect observed here is that under conditions of carbon limitation, the plasmid-containing strain grows more slowly than the plasmid-free strain. However, at high density, the plasmid-containing strain produces sufficient colicin to depress the growth rate of the plasmid-free

TABLE 2

Growth rates

Bacterial strain	Specific growth rate (hr^{-1}) ± SEM*	Doubling time (min)
RH202	0.839 ± 0.026	50
RH250	0.850 ± 0.055	49
RH256	0.768 ± 0.042	54
RH268	0.781 ± 0.056	53

* Estimates are reported together with the standard error of the mean. Standard errors were calculated using standard regression procedures, which assume that the individual errors for each measurement are uncorrelated and identically distributed with mean zero and variance, σ^2. Although this assumption is not strictly correct for the growth equation, the standard errors may be considered a good first approximation to the true standard errors.

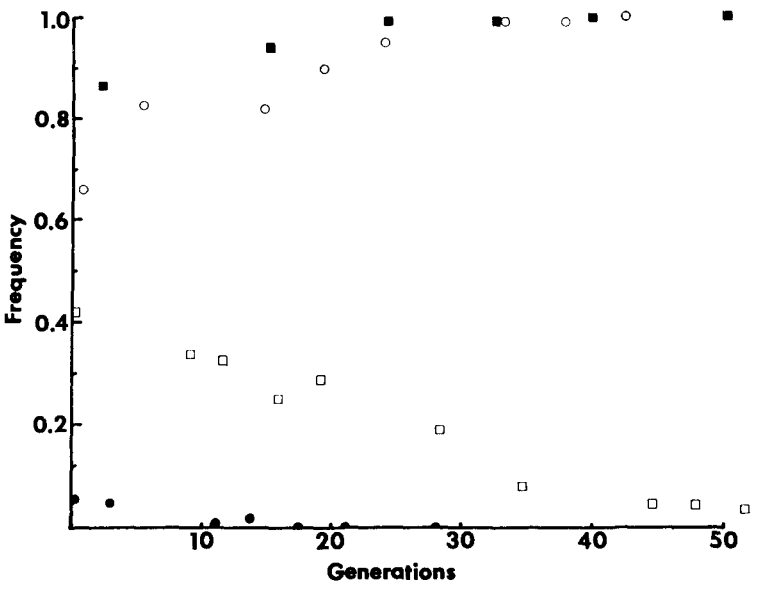

FIGURE 1.—Change in the frequency of RH250 in mixed populations of RH250 and RH202 in glucose-limited chemostats. Each different symbol represents an experiment with a different initial frequency of RH250.

strain. Optimal colicin production apparently requires the presence of glucose in the growth medium (NAKAZAWA, SUZUKI and TAMADA 1977).

These premises predict that the frequency-dependent effect will be lost if the plasmid-free cells become immune to colicin or, alternatively, if the plasmid-containing cells lose their ability to produce colicin. The results of the following experiments are in accord with these predictions.

The first experiment showed that plasmid-free cells immune to colicin would possess a selective advantage regardless of their initial frequency in the chemostat. A glucose-limited chemostat culture was initiated with a high frequency of plasmid-containing cells (RH250). The plasmid-containing cells increased in frequency as in the experiments described previously (Figure 1). However, the plasmid-free cells (RH202) were not eliminated completely from the chemostat, but were maintained in low frequency (<0.01). Two factors contributed to this maintenance: (1) growth of a small number of cells on the wall of the culture vessel, preventing complete elimination, and (2) the constant production of RH202 cells by low-frequency segregation loss of plasmids from RH250 cells. Under such conditions, colicin-immune mutants would be selected for in the residual sector of the population that is plasmid-free. Figure 2 shows that after 100 cell generations the plasmid-bearing cells began to be replaced by plasmid-free cells, which increased in frequency until the experiment was terminated approximately 40 generations later. Ampicillin-sensitive cells were isolated at generation 125 (time point 2, Figure 2) and tested for immunity to colicin. As expected, all ampicillin-sensitive cells (ten of ten tested) were colicin immune.

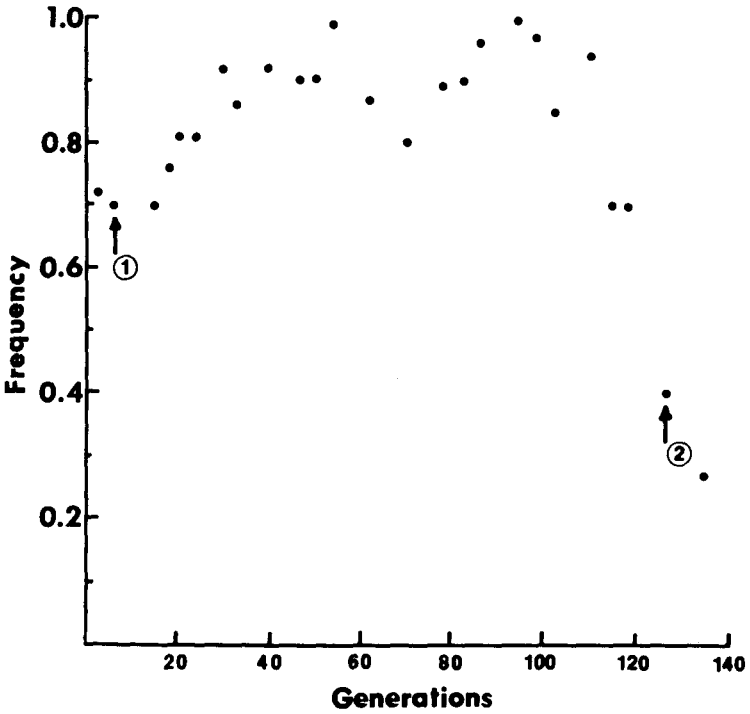

FIGURE 2.—Change in the frequency of RH250 in a mixed population of RH250 and RH202 in a glucose-limited chemostat. RH202 was tested for colicin sensitivity at generation five (time point 1) and at generation 125 (time point 2).

Three of these were tested and found to lack any detectable plasmid DNA. In comparison, ampicillin-sensitive cells isolated from the beginning of the experiment (time point 1, Figure 2) were all colicin sensitive. These results indicate that the frequency-dependent effect disappears, and the plasmid-free cells are selected for independent of the initial frequency, provided they are resistant to ColE1.

A second series of experiments tested the prediction that plasmid-containing cells that did not produce colicin would always be selected against and there would be no frequency-dependent effect.

The plasmid RSF2124 has a single restriction site for the enzyme EcoRI (So, GILL and FALKOW 1975). Recombinant plasmids that contain DNA inserted at this site do not produce colin. It would therefore be expected that the relative fitness of strains containing RSF2124 modified in this way would not be frequency dependent. Cells containing the derived plasmid pML31 were grown to numerical equilibrium in a glucose-limited chemostat and plasmid-free cells (RH202) were then added such that their initial frequency was less than 0.01. Figure 3 shows the change in the frequency of the strain carrying pML31 over time in a typical experiment. It can be seen that the plasmid is lost from the population and the loss is independent of the initial frequency of the plasmid-

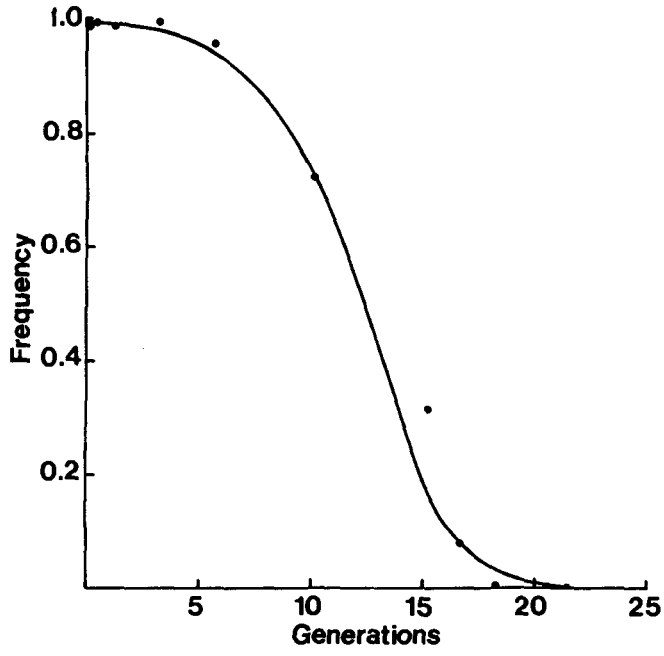

FIGURE 3.—Change in the frequency of RH268 in a mixed population of RH202 and RH268.

containing strain (RH268). To ensure that this loss of the frequency-dependent effect was not unique to cells containing plasmid pML31, the experiment was repeated using a strain carrying a second plasmid, pML27 (RH256). The

Loss of plasmid-containing cells from the population is due to their reduced growth rate: There are two possible explanations for the loss of the plasmid from the population. (1) Plasmid-free cells are generated from the plasmid-containing cells at a high rate, either because of faulty segregation at cell division or because the plasmid is destroyed. Thus, the results shown in Figures 3 and 4 could be explained by an increased rate of segregation of the derived plasmids and not by lack of the frequency-dependent effect observed with RSF2124-containing cells. (2) The plasmid reduces the efficiency with which the host cell can utilize limiting glucose and thus depresses the growth rate of the bacterial host cells. To distinguish between these two hypotheses, the experiments shown in Figure 4 were repeated using a plasmid-free strain marked with an *araAh9* mutation (RH204). This strain was constructed by transduction with P1 and thus is considered to be completely isogenic to RH202 with the exception of the *ara* mutation. Use of this strain allowed the unambiguous identification of ampicillin-sensitive cells derived by the segregated loss of the pML27 plasmid (Ara+). The results are shown in Figure 5A. As before, the plasmid-containing strain results shown in Figure 4 are a composite of three experiments. They illustrate the same loss of the frequency-dependent effect shown in Figure 3 for pML31. is selected against, starting from an initial frequency of close to 1.0. The rates of increase of the two plasmid-free strains (Ara- and Ara+) are virtually identi-

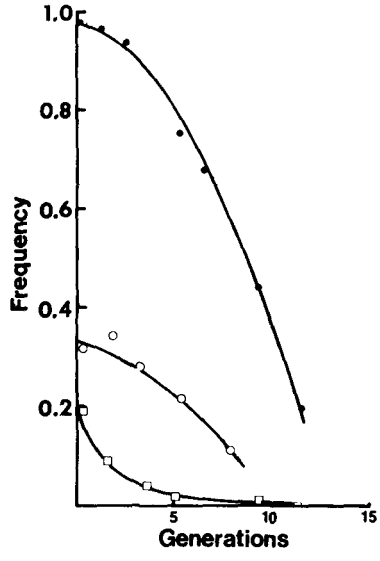

FIGURE 4.—Change in the frequency of RH256 in mixed populations of RH202 and RH256. Each curve represents an experiment with a different initial frequency of RH256.

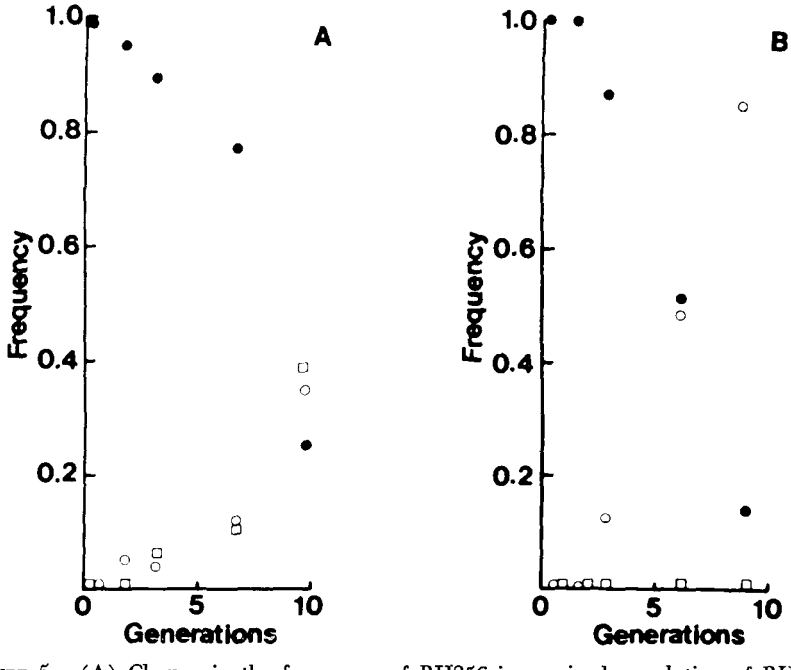

FIGURE 5.—(A) Change in the frequency of RH256 in a mixed population of RH204 and RH256, and the appearance of segregants of RH256 that have lost the plasmid pML27; ●——● frequency of RH256; ○——○ frequency of RH204; □——□ frequency of RH202 obtained by loss of pML27 from RH256. (B) Change in the frequency of RH251 in a mixed population of RH204 and RH251; ●——● frequency of RH251; ○——○ frequency of RH204; □——□ frequency of RH202 (~ 0.0) obtained by loss of plasmid from RH251.

cal, indicating that their fitnesses are essentially the same relative to each other. This demonstrates that although segregational loss of the plasmid occurs, plasmid-containing cells are selected against by virtue of their slower growth rate in comparison to plasmid-free cells. Proof that the nonplasmid-containing cells have a true growth advantage is seen more clearly in Figure 5B. This experiment is similar to that shown in Figure 5A, except that the plasmid employed (pML17) segregates at a very low frequency relative to pML27. As before, the added plasmid-free strain replaced the strain containing the plasmid and segregation was not a complicating factor.

Comparison of the curves shown in Figures 3, 4, and 5 suggest different rates of loss of the strains carrying pML17, pML27 and pML31. The overall relative fitness of each strain is determined by two factors, rate of segregation loss and effect of the plasmid on cellular growth rate. It is reasonable that the magnitude of each of these components will be dependent on what genes are in the inserted piece and also on the length of that piece. The rate of segregational loss of pML27 is greater than that of either pML17 or pML31. This increased rate can be attributed to the particular genes in the cloned DNA segment and not to the size of the inserted fragment because pML27 is intermediate in size between pML17 and pML31. A theoretical treatment of plasmid loss is presented in ANDERSON and LUSTBADER (1975).

DISCUSSION

The results presented in this paper demonstrate that selection for plasmid-containing strains of *E. coli* can be frequency dependent, and that the bases for this frequency dependency are the production of colicin at high density and a lower efficiency of growth on limiting glucose. Similar results have been shown by ZAMENHOF and ZAMENHOF (1971), though these authors did not show conclusively that the frequency dependency was due to bacteriocin production. Our results have implications regarding the maintenance of genetic variation in plasmid populations, and it is worthwhile to examine them in some detail.

It is conventional in eukaryotic population genetics to focus attention on the genetic variation within the population. This is a result of the sexual nature of diploid populations and the predominance of the diploid phase of the life cycle, which allows the possibility of some form of heterozygous advantage. Prokaryotic populations, of course, do not generally possess these characteristics, and with large undefined populations of clonal nature it is more relevant to consider the genetic variation between populations rather than within populations. In this case, clearly, the type of frequency-dependent selection described here will not maintain a genetic variation within populations, as the equilibrium generated by the opposing effects of growth rate and colicin production is unstable. However, this type of selection does suggest a mechanism for the maintenance of genetic variation between populations. Plasmid-containing cells in the population may have a frequency of 0% or 100%, depending on the characteristics of the founding population and also on the environmental conditions. In the environment of the glucose-limited chemostat, cells containing the plasmid RSF2124

increased in frequency whenever their initial frequency was above the critical level of about 50%. This critical frequency will undoubtedly change for different plasmids and for different environments. For those environments that include colicin production to a greater extent, or maintain a higher density of cells, the critical frequency will be lowered. Thus, this frequency-dependent type of selection will also be density dependent. Subsequent experiments (not shown) have confirmed this prediction.

Our results suggest that one mechanism maintaining certain plasmids in bacterial populations may be the selective advantage of colicinogenic plasmids in environments where the concentration of colicin is above a critical level. Resistance to toxic factors such as antibiotic resistance is another well-known selective factor in plasmid maintenance. However, not all plasmids are colicinogenic or induce drug resistance, nor would those properties be advantageous in all environments. It is clear that other mechanisms must be invoked for the maintenance of such plasmids. One possible mechanism may be the possession by many plasmids of insertion elements promoting recombination. Although these elements by themselves may not be selected for, they will increase the genetic lability of the plasmid DNA and facilitate the addition and duplication of bacterial genes on the plasmid. Duplication of a favorable gene on a plasmid would confer a selective advantage to the plasmid and thus to the bacterial cell containing it. In this way plasmids would be selected for much in the same way as mutator genes are selected for in chemostat cultures (GIBSON, SCHEPPE and COX, 1972; PAINTER 1975). If this hypothesis is correct, plasmid-containing cells should eventually replace nonplasmid-containing cells in continuous cultures, even in the absence of colicin production, if a population is followed for a sufficiently large number of generations. The plasmid from these selectively favored cells should have an altered structure reflecting acquisition of rate-limiting genes for growth on low levels of glucose.

These results also have significance to the assessment of risk from cloning DNA by recombinant DNA procedures. RSF2124 is a commonly used cloning vehicle, and any selective advantage possessed by this plasmid might also be conferred on derivatives containing cloned DNA. However, our results show that, even though RSF2124 may be selected for under some conditions, any derived plasmids containing DNA inserts at the EcoRI restriction site will not normally be selected for, because the act of cloning destroys the capacity to produce colicin.

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